ROLE OF ELECTROPHORETICALLY RESOLVED SERUM PROTEIN FRACTIONS FOR THE DIAGNOSIS OF RHEUMATIC HEART DISEASE

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ABSTRACT: In the present study the blood samples of patients diagnosed for rheumatic heart disease (RHD) were obtained from the Punjab Institute of Cardiology, Lahore. Blood samples of the normal subjects of comparable age group with an absent history of cardiac ailment were also collected for the control comparison. The sera of all categories were separated and used for the study of the protein profile with sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in first dimension. Gene Genius Bio-imaging Gel Documentation System was used for the quantification of various protein fractions. This provides the data of molecular weight and percent raw volume for each of the fraction. The protein fractions that showed significant variations were separated by using the technique of electroblotting and electroelution and run on isoelectric focusing (IEF) in second dimension to determine the isoelectric points. In rheumatic heart disease group apolipoprotein B, alpha-2-macroglobulin, ceruloplasmin and immunoglobulin heavy chain were found to be significantly elevated compared to control subjects. A fraction of 23.5 kDa had expressed weakly in 70 % of the patients of rheumatic heart disease but not expressed in control subjects. These results show that level of apolipoprotein B, alpha-2-macroglobulin, ceruloplasmin, immunoglobulin heavy chain and 17 kDa proteins and expression of 23.5 kDa protein fractions are strong predictor of RHD and can be used for its diagnosis.

Key words: Rheumatic heart disease, Protein fractions, Electrophoresis.

INTRODUCTION

Rheumatic heart disease (RHD) remains one of the most important causes of cardiovascular morbidity leading to a major public health problem, especially in developing countries [1] and in Indigenous populations in industrialized countries [2]. Infection with group A Streptococcus is responsible for RHD [3]. It is initiated by streptococcal toxin. The sequence of events almost always begins with a preliminary streptococcal infection caused by specifically by group A hemolytic streptococci, such as sore throat, scarlet fever or middle ear infection. The streptococci release several different proteins against which antibodies are formed, the most important of which seems to be a protein called M antigen. The antibodies then react not only with this M antigen, but unfortunately also with the tissue glycoprotein in the heart, joints and other tissues of the body [4], often causing severe immunological damage [5]. In heart all three layers may be affected [6]. The incidence and mortality rate of rheumatic fever had declined remarkably in many parts of the world over the past thirty years owning to improve socioeconomic conditions, rapid diagnosis and treatment of streptococcal pharyngitis and an unexplained decrease in the virulence of group A streptococci [7]. Improved sanitation and use of antibiotics are also the cause of decline in the incidence of rheumatic fever [6]. In third world countries and in many crowded, economically depressed urban areas in the western world, rheumatic fever remains an important public health problem.

Investigations for diagnosis include throat swab culture for group A streptococcus, elevated erythrocyte sedimentation rate (ESR), C-reactive proteins and prolong PR interval on ECG. During the last few years interest has focused on proteins released into the serum following injury/infection for the diagnosis of cardiovascular disease [8, 9]. Serum haptoglobin was studied in rheumatic heart disease patients by electrophoresis on a cellulose acetate paper and compared it with healthy subjects. It was found that serum haptoglobin was significantly higher in rheumatic heart disease group compared to healthy group [10]. Antitrypsin and antichymotrypsin activity were increased in rheumatic heart disease [11], however, some studies demonstrated normal level of serum alpha-1-antitrypsin in rheumatic heart disease [12]. Increase in inflammatory cytokines was observed in rheumatic heart disease which may use as nonspecific diagnostic methods in assessment of RHD [13]. Elevation of creatine kinase was also showed in eight out of thirty six rheumatic carditis patients [14]. High values of plasma beta-thromboglobulin were also found in rheumatic valvular disease patients [15]. Rheumatic heart disease patients demonstrated higher occurrence of antibodies to cardiolipin [16, 17], however, immunoglobulin G and M antibodies to cardiolipin increased significantly with disease activity [17]. Significant higher concentration of immunoglobulin E and G were observed in patients with rheumatic heart disease than in controls [18].

Applications of newer analytical procedures proceed with the time and one of the useful methodologies involves the electrophoretic system. It is a powerful technique for the analysis of proteins including serum proteins which can be used to study the variations in the protein profiles in RHD patients. Presently two-dimensional gel electrophoresis (2-DE), with one dimension on isoelectric focusing (IEF) and other on sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and vice versa, is mostly employed in such analysis. 2-DE analysis of serum showed a total of 19 spots in the patients of RHD [19]. Two mitochondrial proteins as relevant antigen in rheumatic heart disease in addition to myosin and creatine kinase were identified by two-dimensional immunoblot [20]. These mitochondrial proteins have been implicated in the pathogenesis of inflammatory heart disease for some years. SDS-PAGE is likely to yield protein distribution profile consistent with the patient's clinical status and may have an important role in diagnostic investigation [21].

Present study was carried out to investigate the protein patterns in the sera of RHD patients and their comparison with those of healthy subjects through SDS-PAGE and 2-DE and characterization of the proteins specifically found in the serum of RHD patient in our local population for prediction and diagnosis of RHD.

MATERIALS AND METHODS

Twenty patients from the Punjab Institute of Cardiology, Lahore, with a diagnosis of RHD (based upon throat swab culture for group A streptococcus, elevated erythrocyte sedimentation rate and prolong PR interval on ECG) were selected and their blood samples were collected. Five ml blood was collected from each patient with the help of syringe. Blood samples of same number of healthy subjects with negative family history of CVD were also collected for use as controls. Serum was separated by centrifugation and stored at -70 °C until used for analysis. For SDS-PAGE, the serum samples were diluted in phosphate buffer (pH 7.2) and proteins were denatured by heating with loading dye (1.54 g dithiothreitol, 2 g sodiumdodocyle sulfate, 8 mL of 1.0 M Tris HCl; pH 6.8, 10 mL of glycerol and 20 mg of bromophenol blue dye) in boiling water bath for two minutes before loading on the gel. Lypholized mixture of proteins SDS-6H for high (205-45 kDa) and SDS VII-L for low (66-14.2 kDa) molecular weight proteins (Sigma Chemicals) were used as molecular weight markers. It was reconstituted, separately, in 1.5 mL of sample buffer (0.0625M Tris HCl pH 6.75, 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue). Heated in a boiling water bath for 2 minutes and stored in aliquots at -70°C. Polyacrylamide gels, 5 % for high and 12 % for low molecular weight proteins, were prepared [22]. Protein size marker and each of the samples were loaded in separate wells and gels were electrophoresed at 20 mA and 200 volts in a cooling chamber maintained at 4°C. Electrophoresis was stopped, immediately, after dye seemed to diffuse in the buffer in the lower chamber. Following electrophoresis, the 5% gel was stained with coomassie brillient blue for 30 minutes and 12 % gel for two hours. After staining the gels were destained until the clearance of blue background. Protein fractions of different molecular weights were visible in the form of blue bands on a transparent background. Gels were photographed and their images were saved for protein quantification by Gene Genius Bio-imaging Gel Documentation System provides the data of molecular weights against protein markers and the total area covered by each of the protein fractions. The data was employed in finding the enhancement or reduction and the appearance and disappearance of particular protein fractions for comparison of the healthy individuals and RHD patients.

Samples containing significant quantities of desired protein fractions were run on SDS-PAGE and the unstained gel was electroblotted on polyvinylidene diflouride (PVDF) membrane [23]. The required protein band from PVDF membrane was excised and electroeluted [24]. Each of the eluted protein was freeze dried and reconstituted in the buffer when used for isoelectric focusing.10 µL solution D (10 % w/v sodiumdodecyle sulfate in 2.3 % w/v dithioerythreitol) was added to 60 µL eluted protein solution, mixed and heated at 95°C for 5 minutes. Brought to room temperature and added 5 µL solution E (8 M urea, 4 % CHAPS (3-[(3cholamidopropyl) dimethylammonio]-1-Propanesulfonate), 40 mM Tris HCl and 65 mM dithioerythretol, traces of bromophenol blue). The eluted protein was subjected, afterwards, to isoelectric focusing [25] in order to determine its isoelectric point/s against isoelectric focusing markers.

Table 1: Average raw volumes (%) exhibited by electrophoretically separated serum protein fractions of control and rheumatic heart disease (RHD) groups and their percentage differences.

Molecular weight of proteins (kDa)	Average raw volume (%) of protein fractions in control group	Average raw volume (%) of protein fractions in RHD group	Percentage difference in protein fractions in RHD group
270	2.45 ± 0.21	3.27 ± 0.21	34** ↑
190	7.37 ± 0.30	10.21 ± 0.41	39** ↑
186	3.50 ± 0.26	3.75 ± 0.15	07 ↑
135	4.25 ± 0.23	5.40 ± 0.14	27** ↑
115	5.23 ± 0.37	5.62 ± 0.62	08 ↑
100	3.25 ± 0.24	4.12 ± 0.25	27* ↑
77	10.21 ± 0.23	10.60 ± 0.25	04 ↑
66	26.52 ± 0.18	26.72 ± 0.16	01 ↑
54	14.55 ± 0.31	14.66 ± 0.19	01 ↑
45	10.64 ± 0.41	10.45 ± 0.27	02 ↓
36	2.22 ± 0.12	2.15 ± 0.18	03 ↓
28	9.48 ± 0.48	9.64± 0.32	02 ↑
24	12.42 ± 0.29	12.58 ± 0.18	01 ↑
23.5	-	1.51 ± 0.16	-
23	3.38 ± 0.25	3.44 ± 0.20	02 ↑
17	1.34 ± 0.05	1.92 ± 0.26	43* ↑
14	0.96 ± 0.11	1.11 ± 0.18	16 ↑

↑ Increase; ↓ Decrease; * P<0.05; ** P<0.01

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RESULTS

Most of the protein fractions expressed significantly greater in their intensities in RHD group compared to the control group. However, fraction of 23.5 kDa had expressed weakly in fourteen out of the total twenty patients which had no expression in control subjects.

The fractions of 270, 190, 135 and 100 kDa were found to

be significantly elevated by 34 %, 39 %, 27 % (P<0.01) and 27 % (P<0.05) respectively in this group compared to control group. The average raw volume values of these fractions in rheumatic heart disease group were 3.27 ± 0.21 %, 10.21 ± 0.41 %, 5.40 ± 0.41 % and 4.12 ± 0.25 % respectively. The percent raw volumes exhibited by these fractions in control group were 2.45 ± 0.21 %, 7.37 ± 0.30 %, 4.25 ± 0.23 % and 3.25 ± 0.24 % respectively. Amongst low molecular weight protein fractions, it is notable that a fraction of 23.5 kDa had expressed weakly in fourteen out of the total twenty patients, however, this fraction had no expression in control subjects. Protein fraction of 17 kDa although expressed significantly greater by 43 % (P<0.05), however, no appreciable increase was observed if two sample values of patients were excluded, which showed markedly high values compared to all other patients of this group (Table-1; Figure 1-2).

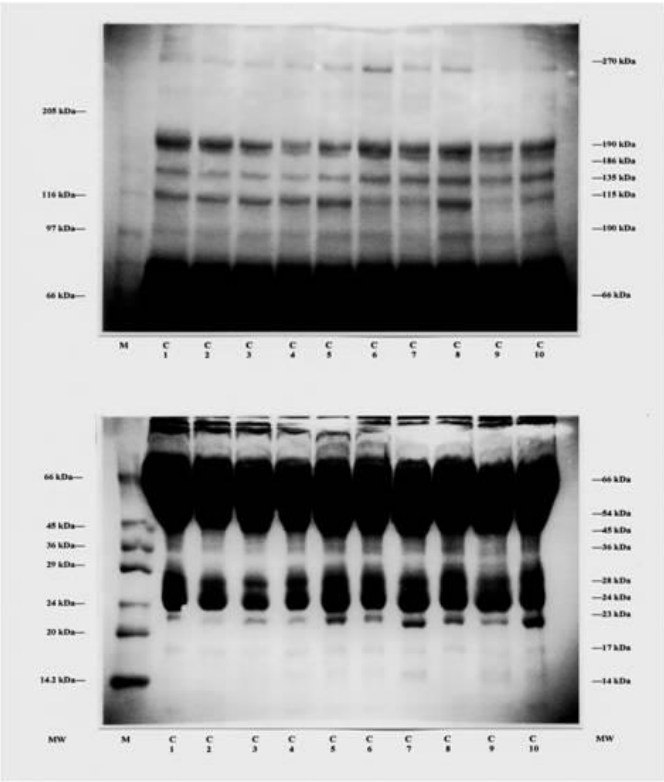

Fig. 1: SDS-PAGE of serum of control subjects. (M indicates protein markers; MW indicates molecular weight of protein markers on left side and serum protein fractions on right side; 5% gel above and 12% gel below).

The protein fractions showing considerable variations were subjected to isoelectric focusing. High molecular weight protein fraction of 270 kDa was resolved into two bands corresponding to isoelectric points 5.9 and 6.1. The fraction of 190 kDa was resolved into five bands whose isoelectric points were determined as 5.4, 5.5, 5.7, 5.9 and 6.1. The fraction of 135 kDa was resolved into two bands whose isoelectric points were determined as 4.8 and 5.2. Lastly the fraction of 100 kDa was resolved into six fractions corresponding to isoelectric points 7.1, 7.2, 7.5, 7.7, 7.8 and 7.9 (Fig. 3).

From the above data regarding the molecular weight and the isoelectric points, each of the proteins was then identified using human plasma protein map [26]. Protein fractions of 270, 190, 135 and 100 kDa were found to be apolipoprotein B, alphs-2-macroglobulin, ceruloplasmin and immunoglobulin heavy chain respectively.

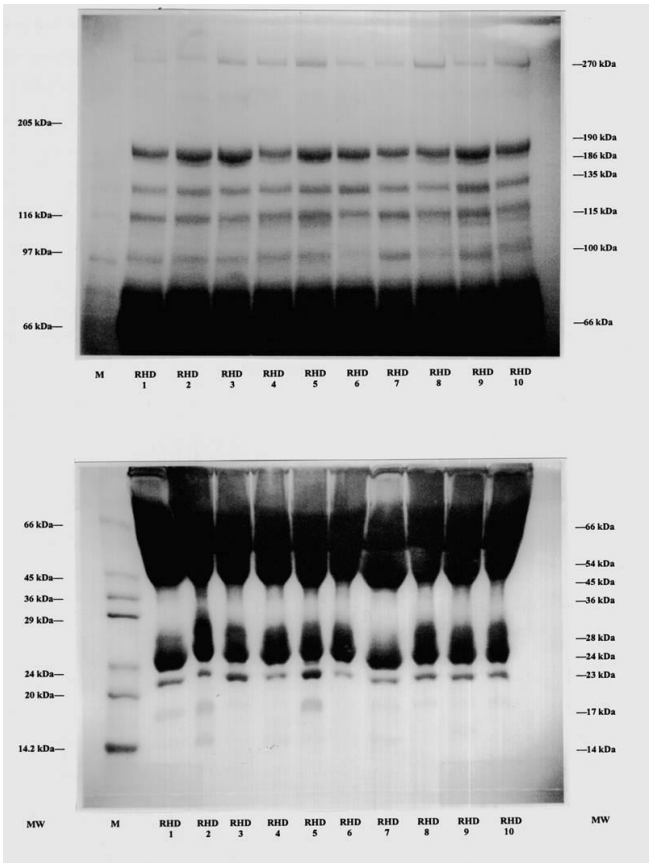

Fig. 2: SDS-PAGE of serum of RHD patients. (M indicates protein markers; MW indicates molecular weight of protein markers on left side and serum protein fractions on right side; 5% gel above and 12% gel below).

DISCUSSION

The aim of the present study was to find the variations in the serum protein profile in the patients of rheumatic heart disease in local sampled population because in recent years proteonomics is a rapidly growing research area and has increased the understanding of many diseases and protein composition represents the functional status of biological compartment. Due to resolution and sensitivity the technique of two- dimensional gel electrophoresis (2-DE) is powerful tool for the analysis and detection of protein from complex biological sources [25]. Variations in the serum protein profile in RHD were therefore detected by sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in first dimension. Isoelectric focusing (IEF) was performed, in second dimension electrophoresis (2-DE), of those proteins which exhibited significant variations and could be diagnostically significant in identifying RHD.

In present study apoplipoprotein B (270 kDa) have been found to be elevated by 34 % in patients of rheumatic heart disease. The excess circulatory levels of any lipoprotein can be caused by one or two factors, either excess production or

decreased catabolism [27]. It is caused by increased apolipoprotein B synthesis by the liver [28, 29] and also due to defect in low density lipoprotein (LDL) receptor, leading to inadequate hepatic uptake of LDL and markedly increasing circulatory LDL or apolipoprotein B, a component of LDL [30]. An important reason of increased LDL level is defective apolipoprotein B in which a substitution of glutamine for arginine at position 3500 results in a form of apolipoprotein B that binds poorly to the receptor and result in reduced LDL clearance and increase LDL or apolipoprotein B concentration in blood [31].

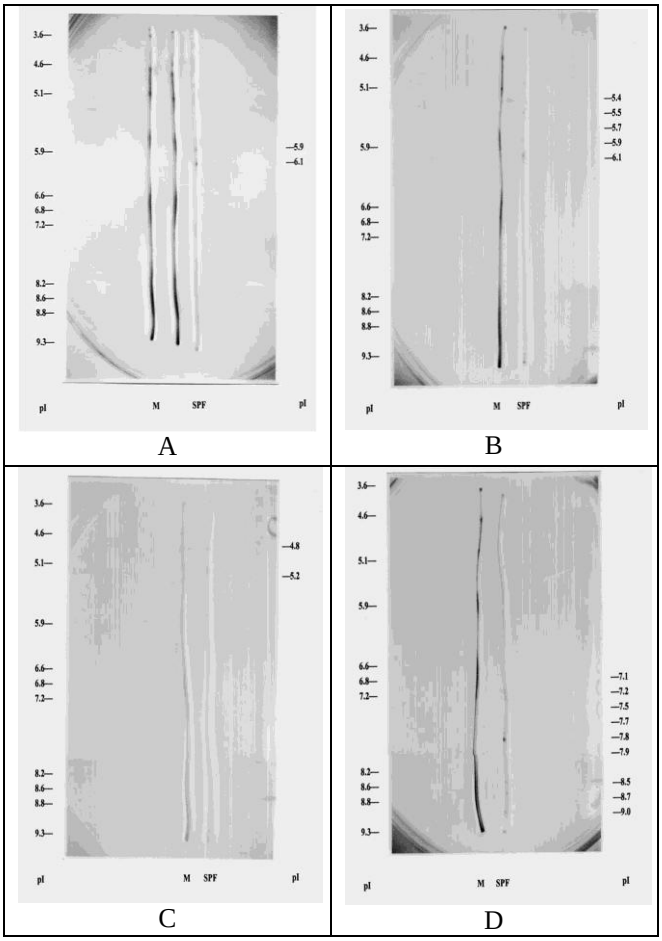

Fig. 3: IEF gels showing distinct bands of 270, 190,135 and 100 kDa protein fraction in Fig. A, B, C and D respectively. (M indicates protein markers and SPF indicates serum protein fractions. pI indicates isoelectric points of protein markers on left side and serum protein fractions on right side).

Another mutation of apolipoprotein B in which a substitution of cystine for arginine at position 3531 impairs binding of apolipoprotein B to the LDL receptor [32]. It is speculated that number of LDL receptor is not fixed and modified by genetic defects, dietary intake of saturated fat, cholesterol and calories and certain pharmacological agents. Thus the interaction of genetic and environmental factors control the number of lipoprotein receptors. These interactions may explain different responses within populations to similar dietary constituents [27, 33]. High use of alcohol [34] and hypertension [35] also the cause of increased level of apolipoprotein B. Smoking [36, 37] increase concentration of LDL and testosterone increase LDL-cholesterol in blood [38]. From the above discussion it is suggested that the higher level of apolipoprotein B may be used as diagnostic marker for cardiovascular disease. It is also indicated that the factors responsible for the enhancement of apolipoprotein B are genetic, dietary and environmental. Management of these factors may be helpful in reducing the chances of the disease.

In human plasma apolipoprotein B occurs in two forms apolipoprotein B-100 and apolipoprotein B-48, which are derived from a single gene [39-43] on the short arm of chromosome 2. Apolipoprotein B-100 is synthesized in the liver, it serves as a structural protein of very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and is the exclusive protein constituent of low density lipoprotein (LDL). Each VLDL, IDL and LDL particle containing one molecule of apolipoprotein B-100. In addition to its structural role, apolipoprotein B-100 functions as a ligand for the LDL receptor. Apolipoprotein B-48 cannot bind to the LDL receptor because it lacks the carbonyl terminal domain of apolipoprotein B-100 [44]. Apolipoprotein B-100 and apolipoprotein B-48 play critical role in the biosynthesis of apolipoprotein B containing lipoproteins. Apolipoprotein B-100 is the major protein moiety of LDL and is responsible for directing the clearance of this lipoprotein through the LDL receptor pathway. Overexpression of apolipoprotein B in transgenic mice increase the levels of LDL and other apolipoprotein B containing lipoproteins [45-50].

An elevation of 39 % was exhibited by alpha-2-macroglobulin (190 kDa) in patients of rheumatic heart disease. Previous studies demonstrated that alpha-2-macroglobulin has a vital but unclear role in immunological and inflammatory process [51]. Alpha-2-macroglobulin is protease inhibitor and synthesized in liver. It has a vital but unclear role in immunological and inflammatory processes. Some evidence suggests that hepatic synthesis of alpha-2-macroglobulin increase in order to compensate partially for the decrease in albumin normally active in maintaining oncotic pressure. Increase level of alpha-2-macroglobulin is also associated with estrogen stimulation as in pregnancy or the use of oral contraceptives [51].

Ceruloplasmin (135 kDa) was found to be elevated by 27 % in patients of rheumatic heart disease. Previous reports also showed higher concentration of ceruloplasmin in patients of cardiovascular disease [52]. This elevated level of copper may suggest the increase in the concentration of ceruloplasmin which is a copper carrying protein. Ceruloplasmin level also rises in disorders producing inflammation or tissue injury. Estrogen reacts with steroid receptors on Hepatocyte to stimulate ceruloplasmin synthesis and plasma level rise substantially in pregnancy or where women take oral contraceptive agents containing estrogen [53].

Ceruloplasmin is copper carrying protein which functions as a ferroxidase and superoxidase scavenger. It is an acute phase protein. The term "acute phase response" encompasses a complex range of physiological changes that occur following infection, inflammation and related conditions. Increase occurs in plasma concentration of ceruloplasmin as a result of increased synthesis, mediated primarily by interleukin-6 and other cytokines. Cytokines

secreted by cells involved in inflammation and immunity [54]. It has been reported that ceruloplasmin may prevent lipid peroxidation and free radical production in inflammatory state [51]. This is perhaps its role in an acute phase reaction.

Immunoglobulins heavy chain (100 kDa) exhibited higher raw volumes by 27 % in patients of rheumatic heart disease. A previous study demonstrated 39 % increase in the concentration of immunoglobulin G (1978 vs. 1429 mg/100 mL) in a group of children with rheumatic heart disease compared to controls. In addition serum immunoglobulin G was also found to be 69 % higher (2814 vs. 1666 iu/mL) in rheumatic heart disease group compared to controls. Higher concentration of immunoglobulin suggested that rheumatic heart disease is complication of infection [18]. In another study elevated serum immunoglobulin G level was noted in patient of rheumatic heart fever as well as inactive rheumatic heart disease [55]. The mean level of total immunoglobulin E in the sera of patients with active rheumatism was significantly higher than during non active phase of the disease and in healthy blood donors and it may have supplementary diagnostic criterion [56]. Measurement of antigen in A, G and M classes immunoglobulins in patients of rheumatism showed higher informative value. Their alterations are to large extent dependent on the pattern of the pathologic process [57]. Increase in serum immunoglobulins are the normal response to infection and chronic bacterial infection cause an increase in serum levels of all immunoglobulins [51].

Immunoglobulins are a group of plasma proteins that function as antibodies, recognizing and binding foreign antigens by elements of the cellular immune system. Since every immunoglobulin molecule is specific for one antigen, so there are vast number of different immunoglobulins. All show a similar basic structure. On electrophoresis immunoglobulin behave mainly as gamma globulins (heavy chain) but immunoglobulin A and immunoglobulin M may migrate with beta or alpha-2-globulins, because the normal plasma concentration of immunoglobulin G is much higher than that of other immunoglobulins. The gamma globulin band seen on electrophoresis of normal serum is largely due to immunoglobulin G. Increases and decreases of plasma immunoglobulin concentrations can be either physiological or pathological in origin. Increased concentrations of immunoglobulin are seen in both acute and chronic infection. Increases in plasma immunoglobulin concentrations are common in autoimmune disease, for example rheumatoid disease and in chronic liver disease, some of which have an autoimmune basis. Many different immunoglobulins are produced in these conditions and they give rise to a diffuse increase in the gamma globulin (heavy chain) band on electrophoresis [40].

Analysis of results of present study indicated that alteration in some protein fractions and week expression of 23.5 kDa protein are indicator of RHD. However the present study is preliminary study and further investigation on large population is required to provide clear situation for the judgment in accessing the protein fraction as marker protein for the diagnosis of RHD. It is also concluded that the technique of electrophoresis, particularly two-dimensional gel electrophoresis, is very useful for the diagnosis of RHD in our Pakistani population where the work on the diagnosis of cardiovascular disease is merely conventional and highly underdeveloped.

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